RESEARCH PAPER

Artificial DNA: PNA & XNA 2:3, 79-89; July/August/September 2011; © 2011 Landes Bioscience

PNA HyBeacons for analysis of human mutations related to statin-induced myopathy

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Key words: PNA, HyBeacon, fluorescence melting, genetic analysis, SNPs, statin-induced myopathy

Abbreviations: Boc, *tert*-butyloxycarbonyl; [t]Bu, *tert*-butyl; CuAAC, copper (I) catalysed alkyne azide cycloaddition; DCM, dichloromethane; DIPEA, diisopropylethylamine; DMAP, 4-diamethylaminopyridine; DMF, *N,N*-dimethylformamide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EDTA, ethylenediaminetetraacetic acid; FAM, fluorescein; HATU, *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate; HBTU, *O*-benzotriazole-*N,N,N',N'*-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenztriazole; hr, hour; NHS, *N*-hydroxysuccinimidyl; PCR, polymerase chain reaction; Piv, pivaloyl; PNA, peptide nucleic acid; PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; RP HPLC, reversed-phase HPLC; rt, room temperature; SNP, single nucleotide polymorphism; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TIS, triisopropylsilane; TLC, thin layer chromatography; T_m, melting temperature; Tosyl, *p*-toluenesulfonyl; UV, ultraviolet

Aminoalkyl and alkyne-tagged PNA HyBeacons have been synthesized, labeled with fluorescein via conventional amide bond or triazole formation (click chemistry) and used to detect single nucleotide polymorphisms (SNPs) implicated in statin-induced myopathy. The PNA HyBeacons gave much better mismatch/mutant discrimination than conventional DNA HyBeacons but smaller fluorescence changes on melting.

Introduction

Fluorogenic PCR[1] using Taqman probes,[2] Scorpions,[3] and Molecular Beacons[4] is an important technology in genetic analysis and diagnostics. Recent developments in high resolution melting analysis promise to revolutionise the field by adding an extra level of information,[5] hence probe-based methods that are fully compatible with this technique are becoming increasingly important. HyBeacon probes[6] (**Fig. 1**) are ideally suited for post-PCR melting analysis as their fluorescence emission changes greatly on duplex melting. Moreover, in the context of melting analysis, HyBeacons have advantages over other probe based methods as they are devoid of secondary structure.

Methods to increase the difference in thermodynamic stability between fully matched and mismatched probe-target duplexes[7,8] are particularly valuable in forensic analysis, genetic testing and SNP detection by melting analysis. As PNA is known to give better mismatch discrimination than DNA,[9] we decided to synthesise and evaluate PNA HyBeacons. A convenient synthesis of fluorescein PNA HyBeacons was developed using two labelling strategies (**Fig. 2**); click chemistry, the copper-catalyzed azide-alkyne cycloaddition (CuAAC reaction), and amide bond formation. Click chemistry[10] has previously been used to label PNA,[11] and is recognised for its high efficiency, whereas amide bond formation is a conventional technique for labelling amino-functionalised DNA[12,13] and PNA. The major advantage of click chemistry over other labelling methods is the very high reactivity of alkynes with azides, and their lack of reactivity with almost all other functional groups.

In this paper we describe an effective synthetic route to modified alkyne and amine-modified PNA monomers **1** and **2** and azidohexylfluorescein **3**. In addition, we have evaluated PNA HyBeacons by probing single nucleotide polymorphisms (SNPs) at the Rs4149056 locus of the human genome that are correlated with statin-induced myopathy in high risk individuals.[14]

Results and Discussion

Synthesis of alkyne-T-PNA monomer 1. The synthesis of alkyne-T-PNA monomer **1** was accomplished according to **Scheme 1**. Synthesis of the PNA backbone **4** using 2,4-dinitrosulfonamide chemistry was efficient and straightforward.[15,16] Alkylation of iodouracil with ethyl bromoacetate followed by Sonogashira coupling gave compound **6** in 75% overall yield. After hydrolysis of ester **6**, carboxylic acid **7** was coupled to PNA backbone **4** to afford the fully protected PNA monomer **8** in 92% yield. Finally the *tert*-butyl ester of **8** was cleaved in acid to yield the monomer **1** in 78% yield (2:1 rotamers).

Synthesis of aminoalkyl-T-PNA monomer 2 (Scheme 2). The modified base moiety of aminoalkyl-T-PNA monomer **2**

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Submitted: 07/28/11; Revised: 09/21/11; Accepted: 09/22/11
DOI:

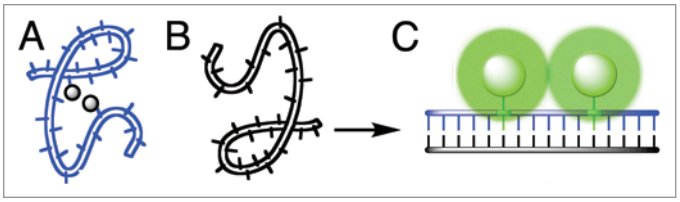


Figure 1. HyBeacon principle: In the unhybridised HyBeacon (A) the two fluorophores (black circles) interact and fluorescence is quenched. Binding to complementary target strand (B) leads to a highly fluorescent duplex (C).

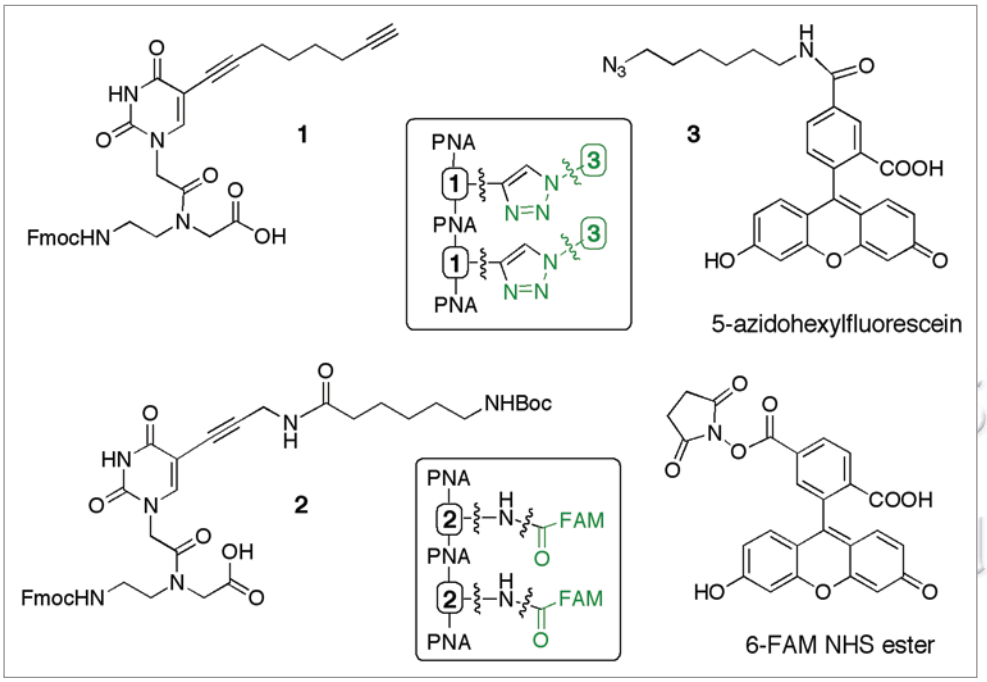
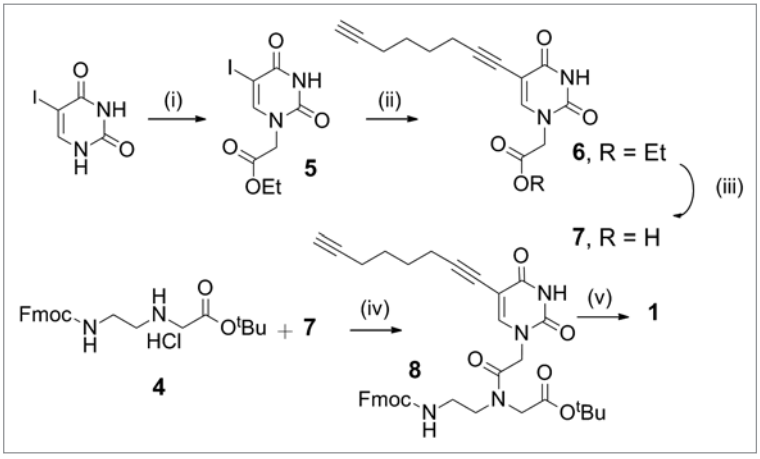


Figure 2. Modified PNA thymine monomers and PNA HyBeacon probes. **1** = alkyne-T-PNA monomer; **2** = aminoalkyl-T-PNA monomer; **3** = 5-azidohexylfluorescein. Boxes: PNA HyBeacon containing triazole and amide bond linkages, FAM = fluorescein.



Scheme 1. Synthesis of alkyne-T-PNA monomer **1**: (i) $BrCH_2CO_2Et$, K_2CO_3, DMF, 2 hr, rt, 89%; (ii) 1,7-octadiyne, CuI, Et_3N, $Pd(Ph_3)_4$, DMF, rt, 2 hr, 82%; (iii) LiOH, 1,4-dioxane/H_2O (2:1, v/v), rt, 30 min, Dowex 50 (H^+), 82%; (iv) HBTU, DIPEA, DMF, 3 hr, 92%; (v) 95:2.5:2.5, TFA:water:TIS (v/v), 30 min, rt, 78%.

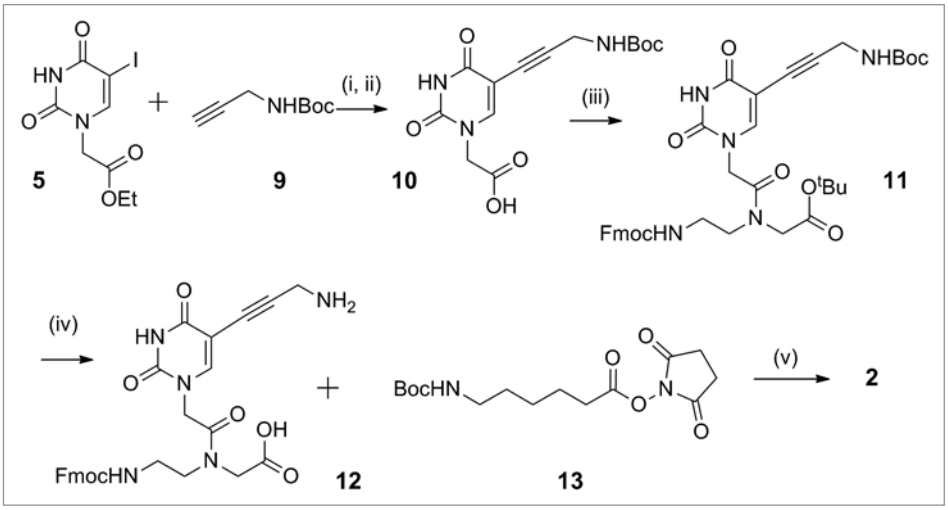
was synthesized from propargylamine, iodouracil and 6-aminohexanoic acid. The first two steps were similar to alkyne-T-PNA monomer **1**, briefly *N*-Boc-protected propargylamine **9**,[17] was coupled to modified iodouracil **5** via a Sonogashira coupling after which hydroysis of the ethyl ester under basic conditions gave the pure product in 82% yield over two steps. Compound **10** was coupled to PNA backbone **4** using PyBOP activator after which the Boc and ^{t}Bu protecting groups of **11** were removed using trifluoroacetic acid to give compound **12**. The free amino group of **12** was then coupled to *N*-Boc-aminohexanoic acid NHS ester **13**,[18] to give the aminoalkyl-T-PNA monomer **2** in 63% yield (2:1 rotamers).

Synthesis of 5-azidohexylfluorescein 3. The synthesis of 5-azidohexylfluorescein **3** was achieved from the inexpensive, commercially-available precursors 6-aminohexanol and 5(6)-carboxyfluorescein in good yield (**Scheme 3**). 6-Aminohexanol was protected at the amino terminus using ethyl trifluoroacetate, followed by conversion of the alcohol to azide via tosyl derivative **15** to give compound **16**. The trifluoroacetyl protecting group at the amino terminus was then removed with aqueous ammonia to give amine **17** which was coupled to the carboxyfluorescein dye **18**,[19] using EDC chemistry giving, after purification, 5-azidohexylfluorescein **19** as a major product (24%) along with 6-azidohexylfluorescein (<5%). Finally, the pivaloyl groups of compound **19** were removed under basic conditions using a mixture of ammonium hydroxide and 1,4-dioxane.

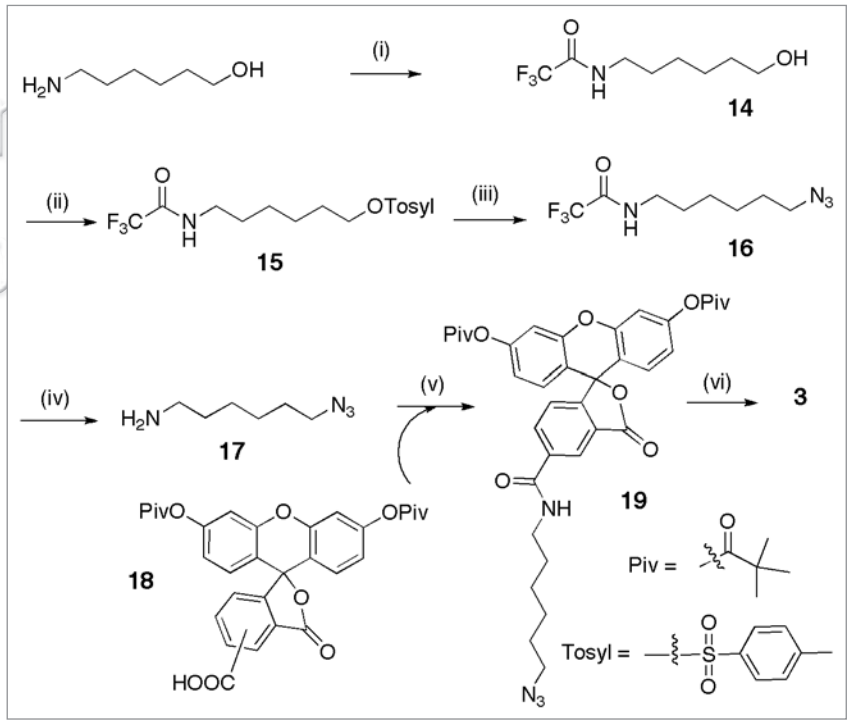
PNA design and synthesis of PNA HyBeacon probes. To investigate the properties of PNA HyBeacons, a series of fluorescently labelled PNA probes for the human Rs4149056 target were synthesised using the following resource: (www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=rs4149056) The wild-type probes (WP) contain thymine at the site of polymorphism and the mutant probes (MP) contain cytosine (**Table 1**).

Each PNA HyBeacon contains two or three fluorescein dyes with different spacings between them. Multiple fluorescent dyes are essential for HyBeacon to produce effective melting curves. PNA probes containing monomers **1** and **2** were synthesised by standard Fmoc solid-phase PNA synthesis methods and conjugation to the corresponding functionalised fluorescein PNAs was performed both in solution and on solid phases via click chemistry and amide bond formation.

In the click fluorescein labelling reaction under both solid and solution phase reaction conditions the ratio of alkyne PNA:5-azidohexylfluorescein **3**:copper sulfate:sodium ascorbate was 1:50:10:100 and in all cases the conversion was more than 80%. Dye-labelling via amide bond formation was carried out by mixing 6-carboxy-fluorescein succinimidyl ester with PNA containing free amino groups on aminoalkyl-T-PNA monomer **2** in a mixture of DMF:aqueous buffer (0.5 M $NaHCO_3/Na_2CO_3$, 1:1, v/v, pH 8.75). The reactions were left at room temperature for 4–6 hr and afforded the products in ~80% yield (calculated by RP-HPLC analysis). The purity of the crude PNA before and after labelling with three units of the modified PNA monomers was slightly lower than with two units. This is a consequence of less than quantitative coupling due to the bulk of fluorescein and the proximity of the modified bases. Example of RP-HPLC traces of PNA before and after labelling with fluorescein (amide bond formation) with three additions of the aminoalkyl-T-PNA monomer **2** and after click labelling with two additions of alkyne-T-PNA monomer **1** in solution phase are shown in **Figure 3**. The RP-HPLC of the crude PNA before labelling with the fluorescent dye shows the purity to be 70–80%. The product distribution after labelling in solution for three additions of the modified aminoalkyl-T-PNA monomer **2** with 6-carboxyfluorescein succinimidyl ester at positions 5, 9 and 12 (WP32Am) was 80% of the desired product and 15% of incomplete labelled products containing two fluorescein units. The click reaction to insert two additions of fluorescein at positions 5 and 12 went almost to completion (>98%, WP6Cl2).



Scheme 2. Synthesis of aminoalkyl-T-PNA monomer **2**: (i) $Pd(PPh_3)_4$, CuI, DMF, Et_3N, 2 hr, 90%; (ii) LiOH, water/MeOH (1:3, v/v), 45 min, rt, then Dowex 50 (H^+)/pyridine, 91%; (iii) PyBOP, HOBt, **4**, DIPEA, DMF, rt, 3 hr, 63%; (iv) 95:2.5:2.5, TFA:water:TIS (v/v), 30 min, rt, 95%; (v) DIPEA, DMF, 4 hr, 63%.



Scheme 3. Synthesis of 5-azidohexylfluorescein **3** (i) CF_3CO_2Et, DMAP, CH_2Cl_2, rt, 1 hr; (ii) tosyl chloride, Et_3N, CH_2Cl_2, rt, 5 hr, 70% for 2 steps; (iii) K_2CO_3, DMF, NaN_3, 50°C, 5 hr, 94%; (iv) 35% NH_4OH, 12 hr, rt, then Dowex 50 (H^+), 76%; (v) EDC, HOBt, rt, 1 hr, 24%; (vi) 1,4-dioxane/NH_4OH (1:2, v/v), rt, 3.5 hr, 93%.

Fluorescence melting studies. Melting analyses (**Table 1**) were performed to evaluate the PNA HyBeacons using wild-type and mutant synthetic DNA targets (Wtt and Mtt) with adenine and guanine respectively at the site of polymorphism. Asymmetric PCR amplification and post-PCR melting analysis was also carried out using a 10:1 ratio of primers RP:FP to produce excess of the target strand for the HyBeacon probes. These experiments were carried out on a Roche LightCycler 1.5 and required a total of 60 min to complete the assay (50 min for PCR and 10 min for melting analysis). The fluorescein PNA HyBeacons with click triazole and amide linkages gave similar T_ms (**Fig. 4**), but the melting peaks of the fluorescein amide PNA HyBeacons were slightly sharper than the triazole analogues. The T_ms of mutant probes where the C base in the probe binds to mutant target (C:G base pair), were much higher (ΔT_m = 17°C) than that of the wild-type target (C:A mismatch). For the wild-type probe with thymine at the point of discrimination (wild-type:target = T:A, mutant:target = T:G) the difference in T_m was 9°C. This is excellent discrimination considering the fact that TA is the

Table 1. PNA and DNA sequences, melting temperatures (T_ms), quantum yields and MS analyses

Oligo ID	Sequence	T_m (°C) Wt(A)	T_m (°C) Mt(G)	ΔT_m (°C)	Quantum yield*[a] ssDNA	Quantum yield*[a] dsDNA	MALDI/ES+ (Da) Found	MALDI/ES+ (Da) Calc.
	HyBeacon Probes							
WP3Am	acG ATA TAf GTG fTC ATG O	60.6	51.3	9.3	0.170	0.238 (T:A)	5750.9	5750.1
WP6Am	acG ATA fAT GTG TfC ATG O	60.4	53.6	6.8	0.155	0.252 (T:A)	5752.7	5751.4
WP6Cl	GAT AYA TGT GTY CAT GKK K	62.0	53.9	8.1	0.061	0.133 (T:A)	5969.0	5969.7
WP32Am	acG ATA fAT GfG TfC ATG O	58.6	54.3	4.3	0.133	0.185 (T:A)	6260.1	6259.3
WP32Cl	GAT AYA TGY GT YCA TGK KK	62.0	56.3	5.7	0.034	0.107 (T:A)	6559.4	6560.8
MP3Am	acG ATA TAf GCG fTC ATG O	48.5	63.3	-14.8	0.279	0.282 (C:G)	5737.5	5735.1
MP3Cl	GAT ATA YGC GYT CAT GKK K	49.0	63.7	-14.7	0.026	0.050 (C:G)	5952.5	5954.7
MP6Cl	GAT AYA TGC GTY CAT GKK K	50.1	64.2	-14.1	0.206	0.223 (C:G)	5955.6	5954.7
MP6Cl2	OAT AYA TGC GTY CAT KK	36.0	53.2	-17.2	0.075	0.169 (C:G)	5535.7	5533.4
WP6Cl2	OAT AYA TGT GTY CAT KK	47.5	38.6	8.9	0.083	0.140 (T:A)	5548.6	5548.4
MP DNA	GTG GAT ATA FGC GFT CAT GGP	43.0	52.0	9.0	0.070	0.204 (C:G)	nd	nd
Wtt	GGA ATC TGG GTC ATA CAT GTG GAT ATA TGT GTT CAT GGG TAA TAT GCT TCG TGG AAT AG							
Mtt	As Wtt template but with C instead of T							
FP	GAA TCT GGG TCA TAC ATG							
RP	TAT TCC ACG AAG CAT ATT							
	PNA before labelling with dye*[b]					ΔT_m *[c]		
WP3U2	acG ATA TA2 GTG 2TC ATG O	67.6	58.9	9.7		7.0		
WP6U2	acG ATA 2AT GTG T2C ATG O	68.1	59.1	9.0		7.7		
WP6U1	GAT A1A TGT GT1 CAT GKK K	67.6	58.9	8.7		8.7		
WP32U2	acG ATA 2AT G2G T2C ATG O	70.3	63.2	7.1		11.7		
WP32U1	GAT A1A TG1 GT1 CAT GKK K	66.6	60.4	6.2		4.6		
MP3U2	acG ATA TA2 GCG 2TC ATG O	58.0	71.2	-13.2		7.9		
MP3U1	GAT ATA 1GC G1T CAT GKK K	55.7	69.4	-13.7		5.7		
MP6U1	GAT A1A TGC GT1 CAT GKK K	57.8	71.7	-13.9		7.5		
MP6U2	OAT AYA TGC GTY CAT KK							
WP6U2	OAT AYA TGT GTY CAT KK							
synth Mt	CCA TGA ACG CAT ATA TCC							
synth Wt	CCA TGA ACA CAT ATA TCC							

WP & MP = wild-type and mutant probes; Am & Cl = probe labelling via amide bond and click chemistry; Wtt & Mtt = wild-type and mutant templates/targets (Wt) "A" and mutant (Mt) target "G"; FP and RP = forward and reverse primers; U1 & U2 = PNA with alkyne-T-monomer 1 and aminoalkyl-T-monomer 2 before labelling with fluorescent dyes; synth Wt and synth Mt = synthetic wild-type and synthetic mutant targets; ac = acetyl, O = double aminoethoxyethoxyacetyl, K = lysine, f = fluorescein with aminoalkyl-T-PNA monomer 2, Y = alkyne-T-PNA monomer 1 clicked to 5-azidohexylfluorescein 3, F = fluorescein dT (Glen Research), P = phosphate, 1 = alkyne-T-PNA monomer; 2 = aminoalkyl-T-PNA monomer; The SNPs are underlined in blue; ss & ds = single and double stranded DNA, *a = quantum yield is an average of 10 measurements; Calc = calculated mass. T_ms = T_m of the HyBeacon probe was measured with PCR products of Wtt and Mtt whereas T_m of the PNA before labelling was measured with synthetic Mt and Wt targets (*b); ΔT_m = T_m of (Wt-Mt). *c = (T_m of PNA and synthetic target before labelling with dye) - (T_m of PNA and synthetic target after fluorescent labelling).

least stable Watson-Crick base pair and GT is a relatively stable mismatch.

For effective SNP genotyping and mutation detection, the homozygote and heterozygote must be clearly distinguished. This was evaluated by mixing the Wtt and Mtt targets in the PCR reaction and analysing the melting data. Examples of melting peaks of PNA probes hybridized to wild-type, mutant and mixed targets are shown in **Figure 5**. The discrimination between them is very clear. These curves represent the PNA probes bound to the Rs4149056 locus, the black curves show the homozygous alleles (TT and CC) and the red curves repesent the heterozygous allele (TC).

Comparing the ΔT_ms of the duplexes between HyBeacon probes with wild-type target and mutant targets, PNA HyBeacons probes gave much greater ΔT_ms than the DNA HyBeacon probe (up to 8°C difference). However, the fluorescence intensities of the hybridized PNA probes were lower than the DNA HyBeacon probes by up to a factor of four (**Fig. 6**). This is currently a limitation of PNA HyBeacons; the fluorescence change on duplex melting is rather small.

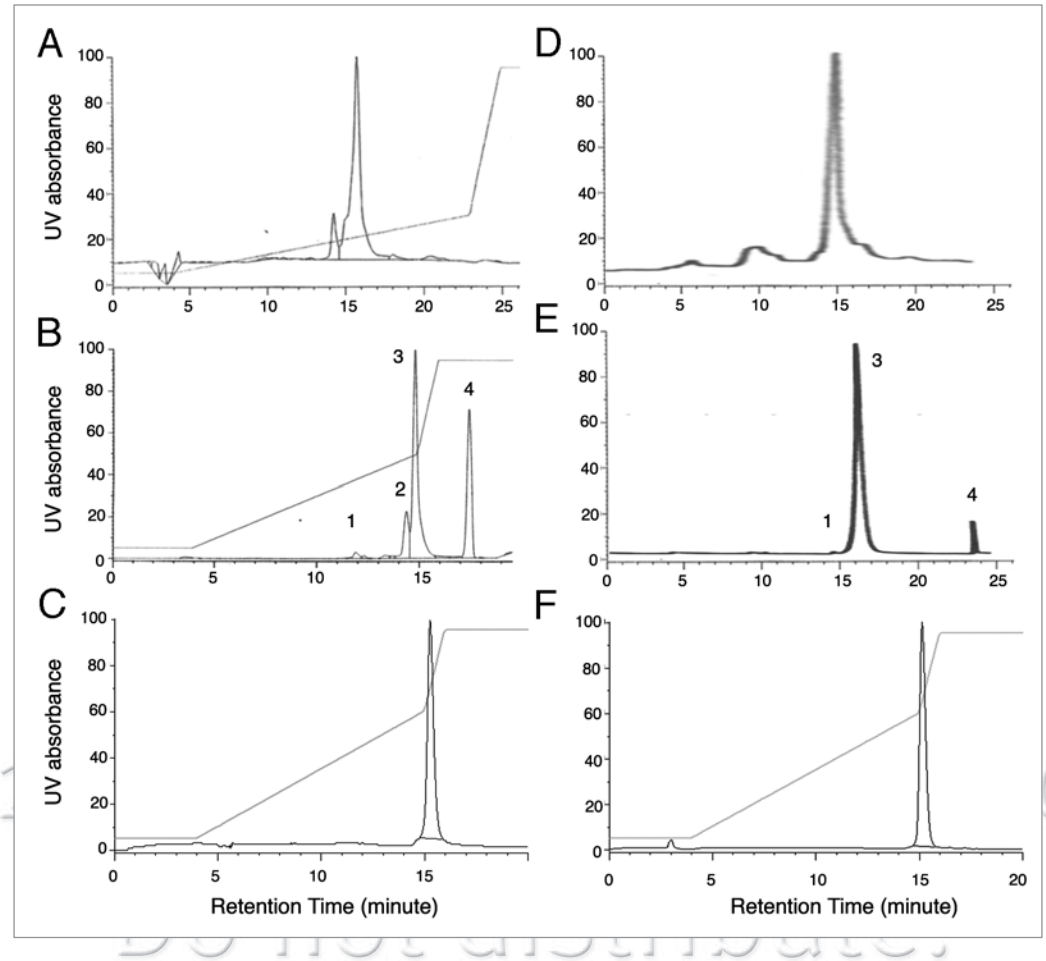


Figure 3. RP-HPLC of PNA probes on C18 column. (A) Unpurified triply amine-labelled WP32U2, (B and C) Unpurified and purified triple fluorescein-labelled WP32Am, (D) Unpurified doubly alkyne-labelled WP6U2, (E and F). Unpurified and purified doubly fluorescein-labelled WP6Cl2 where 1, unlabeled PNA; 2, incomplete labelling; 3, correct product; 4, free dye. (A, B, C and F) UV absorbance at 270 nm (analytical), (D and E) at 300 nm (preparative). A, B, C and F show the gradient of buffer B. Buffer A = 0.1% TFA in water and buffer B = 0.08% TFA in MeCN.

Comparing the intensity of fluorescence of the DNA and PNA HyBeacons (**Fig. 6**), the duplex fluorescence is very similar whereas in the single strand the PNA is twice as fluorescent as the DNA. This indicates that the fluorophores in the single stranded PNA HyBeacon probe are not efficiently quenched. Quantum yields of the single stranded PNA and DNA probes and their duplexes with fully complementary synthetic oligonucleotide targets were determined and the results are shown in **Table 1**. The difference in quantum yield between the single and double stranded DNA probes was greater than for the equivalent PNA, which is in accordance with the fluorescence melting profiles. The melting curves of the PNA probes added before and after PCR amplification gave similar results, indicating that there was no inhibition of PCR by the PNA.

It is noteworthy that the ΔT_ms and melting peak resolution between the matched and mismatched duplexes are much greater for PNA than for DNA probes, a significant factor in clinical diagnostics. Other findings are: (i) the order of stablility of the matched and mismatched PNA probes/targets duplex are C:G>T:A>T:G>C:A confirming that the stability trends for the PNA HyBeacons are similar to their DNA counterparts; (ii) the mismatched PNA and DNA targets generated weaker fluorescence melting transitions than the fully matched systems. (iii) the T_ms of unlabelled PNA (U) were higher than the fluorescently labelled counterparts with an average ΔT_m of 3.3–4.3°C per modified base; (iv) the unlabelled PNA duplex with two and three additions of the aminoalkyl-T-PNA monomer **2** was more stable by 4°C than the unlabelled alkyne-T-PNA duplex containing monomer **1**. This is probably due to the interaction of the positive charge of the protonated amino group and the phosphate backbone of the DNA target. However when the amine is conjugated to the fluorescent dye, the duplex is destabilised; (v) placing two or three lysine residues at the carboxy-terminus of the PNA significantly improves solubility in aqueous media.

Materials and Methods

General. Reactions which required a dry atmosphere were conducted in flame-dried glassware under argon gas. Unless otherwise stated, commercial grade solvents and reagents were used without further purification. Tetrahydrofuran (THF) was

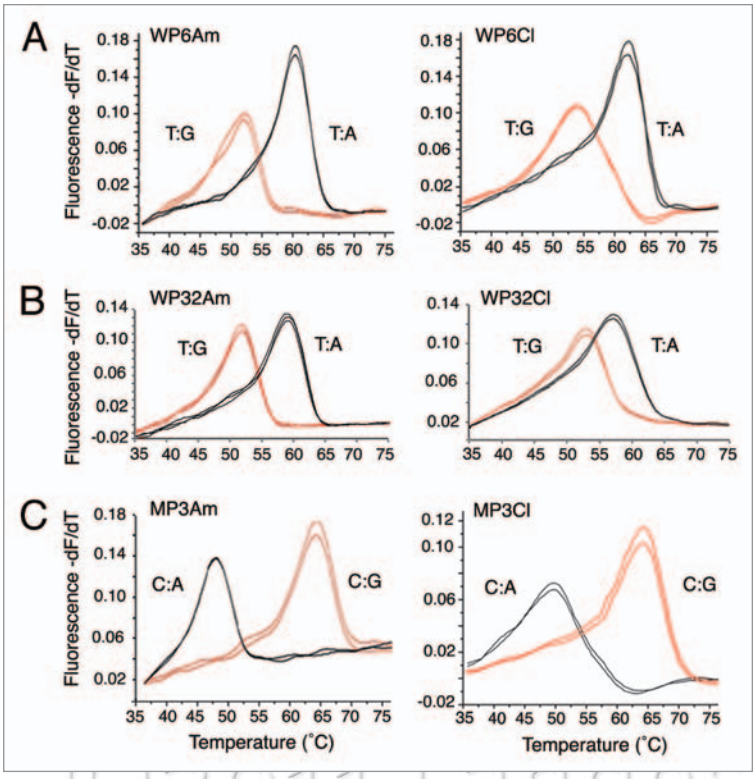


Figure 4. Comparison of melting peaks of duplexes between PNA probes and asymmetric PCR products of Mtt and Wtt oligonucleotide targets. Probes were labelled by amide bond formation (Am) or click chemistry (Cl). Wp = wild-type probe that contains a "T" base at the SNP. Mp = mutant probe that contains a "C" base at the SNP. T:A = wild-type probe/wild-type target, T:G = wild-type probe/mutant target, C:A = mutant probe/wild-type target and C:G = mutant probe/mutant target.

distilled from sodium using benzophenone as an indicator. Triethylamine, pyridine, *N,N* diisopropylethylamine (DIPEA) and dichloromethane (DCM) were distilled from CaH_2. Thin Layer Chromatography (TLC) which was performed on aluminium sheets coated with silica gel (0.22 mm thickness, aluminum backed, Merck) containing fluorescent indicator $60F_{254}$. Visualisation was achieved by UV radiation at 254 nm and in some cases an additional reagent, i.e., ninhydrin for amines, potassium permanganate for unsaturated compounds and Mary reagent for acids. Purification using column chromatography was performed with chromatography grade Silica gel 60A (particle size 35–70 μL and pore size 60 Å). NMR spectra were recorded using a Brüker AV 300 spectrometer or a Brüker DPX400 spectrometer. Proton-proton correlation (H-H COSY), carbon-proton correlation (C-H COSY) and DEPT-135 methods were also used to assign the spectra. Chemical shifts (δ) are given in ppm and *J* values are given in Hz and are correct to within 0.5 Hz. All spectra were internally referenced to the appropriate residual undeuterated solvent signal. The following abbreviations apply: "s" for singlet, "d" for doublet, "t" for triplet, "q" for quarter "br" for broad, "obsc" for obscure and "m" for multiplet. Low-resolution mass spectra (LRMS) were recorded using the electrospray technique on a Fisons VG platform instrument or a Waters ZMD quadrupole instrument. High-resolution mass spectra (HRMS) were recorded using the electrospray technique on a Bruker APEX III FT-ICR mass spectrometer.

ethyl (5-iodouracil-1-yl)acetate, 5. Potassium carbonate (6.28 g, 45.8 mmol) was added to a solution of 5-iodouracil (9.90 g, 41.6 mmol) in anhydrous *N,N*-dimethylformamide (80 mL) under argon, followed by slow addition of ethyl-bromoacetate (4.52 g, 40.8 mmol). The reaction mixture was stirred at room temperature and after 2 h, filtered through a pad of Celite, concentrated by co-evaporation with toluene (20 mL x 3) and purified by precipitation using dichloromethane and diethyl ether to yield the title product as an amorphous white solid (11.79 g, 89%). δ_H (300 MHz, $CDCl_3$) 8.84 (1H, s, N**H**), 7.58 (1H, s, NC**H**), 4.48 (2H, s, NC$\mathbf{H}_2$), 4.31-4.24 (2H, q, *J* = 7.0 Hz, OC$\mathbf{H}_2$), 1.34-1.30 (3H, t, *J* = 7.0 Hz, C$\mathbf{H}_3$); δ_C (75.5 MHz, DMSO-d_6) 167.0 (C), 160.1 (C), 150.2 (C), 148.6 (CH), 68.4 (C), 62.5 (CH_2), 48.8 (CH_2), 14.1 (CH_3); LRMS-ESI$^+$: *m/z* 324.9 $[M+H]^+$ (100%).

ethyl [5-(1,7-octadiynyl) uracil]acetate, 6. To a thoroughly degassed solution of ethyl (5-iodouracil-1-yl) acetate **5** (7.78 g, 24.0 mmol) in anhydrous *N,N*-dimethylformamide (100 mL) was added successively anhydrous triethylamine (10 mL), 1,7-octadiyne (3.82 mL, 36.0 mmol), tetrakis(triphenylphosphine)palladium(0) (2.80 g, 2.4 mmol) and copper (I) iodide (914 mg, 4.8 mmol). The mixture was left to stir under argon. After 2

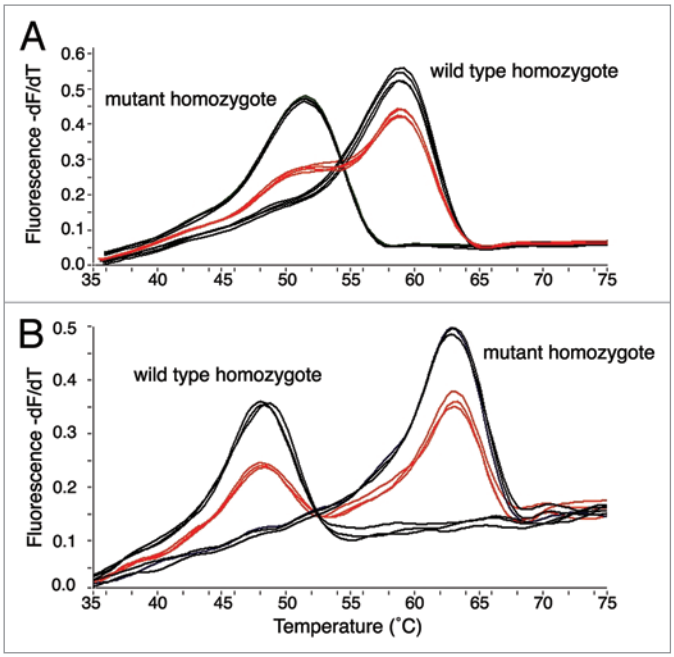


Figure 5. Melting peaks of PNA HyBeacons with asymmetric PCR product of Mtt and Wtt oligonucleotide targets. Individual black melting peaks are homozygotes i.e., probe/wild-type target or probe/mutant target, melting peaks in red show the heterozygotes, i.e., probe bound to mixed targets (both wild-type and mutant). (A) WP32Am: wild-type (T:A), mutant (T:G) and the mixed wild-type and mutant (T:A and T:G). (B) MP3Am: wild-type (C:A), mutant (C:G) and the mixed wild-type and mutant (C:A and C:G).

hr the reaction mixture was poured into a mixture of saturated ammonium chloride (75 mL) and EDTA (Na^+ salt, 4.50 g in water 300 mL pH 8). The mixture was extracted with ethyl acetate (100 mL x 4) and the combined organic extracts were dried over sodium sulfate and filtered. The filtrate was concentrated in vacuo by co-evaporation with toluene (20 mL x 3) and the residue was purified by flash column chromatography eluting with 20% petroleum ether in dichloromethane graded to 2% methanol in dichloromethane, to give ethyl (5-octa-1,7-diynyl)acetate **6** as an amorphous white powder (5.94 g, 82%). δ_H (300 MHz, DMSO-d_6) 11.7 (1H, s, N**H**), 7.98 (1H, s, NC**H**), 4.50 (2H, s, NC$\mathbf{H}_2$), 4.19-4.12 (2H, q, J = 7.0 Hz, OC$\mathbf{H}_2$), 2.77 (1H, t, J = 3 Hz, CC**H**), 2.40 (2H, m, CCC$\mathbf{H}_2$), 2.20 (2H, m, C$\mathbf{H}_2$CCH), 1.57 (4H, m, (C$\mathbf{H}_2$C$\mathbf{H}_2$C$\mathbf{H}_2$CCH)), 1.20 (3H, t, J = 7.0 Hz, C$\mathbf{H}_3$); δ_C (75.5 MHz, DMSO-d_6) 167.8 (C), 162.2 (C), 149.9 (C), 148.2 (CH), 98.2 (C), 92.9 (C), 84.2 (C), 72.5 (C), 71.3 (CH), 61.3 (CH_2), 48.7 (CH_2), 27.2 (CH_2), 27.1 (CH_2), 18.2 (CH_2), 17.2 (CH_2), 14.0 (CH_3); LRMS-ESI$^+$: m/z 342.5 $[M+K]^+$ (100%).

ethyl [5-(1,7-octadiynyl)-uracil]acetic acid, 7. A solution of compound **6** (1.00 g, 3.3 mmol) and lithium hydroxide (0.28 g, 6.6 mmol) in 2:1 1,4-dioxane:water mixture (9 mL) was stirred for 30 min at room temperature. The reaction was diluted with a 1:1 methanol:water (10 mL) and filtered through a pre-washed Dowex 50 (H^+) column. The column was washed with methanol (20 mL) and the combined eluents were concentrated under reduced pressure. The residue was purified by column chromatography using dichloromethane graded to 20% methanol in dichloromethane, to provide the acid 7 as an amorphous white solid (0.74 g, 82%). δ_H (300 MHz, DMSO-d_6) 13.19 (1H, s, COOH), 11.61 (1H, s, N**H**), 7.96 (1H, s, NC**H**), 4.38 (2H, s, NC$\mathbf{H}_2$), 2.76 (1H, t, J = 3 Hz, CH_2CC**H**), 2.39 (2H, m, C$\mathbf{H}_2$CCH), 2.19 (2H, m, CCC$\mathbf{H}_2$), 1.56 (4H, m, C$\mathbf{H}_2$C$\mathbf{H}_2$C$\mathbf{H}_2$CC); δ_C (75.5 MHz, DMSO-d_6) 169.2 (C), 162.3 (C), 149.9 (C), 148.5 (CH), 97.9 (C), 92.8 (C), 84.2 (C), 72.6 (C), 71.3 (CH), 48.8 (CH_2), 27.2 (CH_2), 27.1 (CH_2), 18.2 (CH_2), 17.2 (CH_2); LRMS-ESI$^-$: m/z 273.2 $[M-H]^+$ (100%); HRMS calc. for $C_{16}H_{18}N_2NaO_4$ 297.0851 found 297.0847.

***bis*-protected 5-(1,7-octadiynyl)uracil PNA monomer precursor, 8.** A solution of the acid 7 (1.32 g, 4.8 mmol), HBTU (1.84 g, 4.8 mmol) and *N,N*-diisopropylamine (2.5 mL, 13.9 mmol) in *N,N*-dimethylformamide (6 mL) under argon was stirred at room temperature for 5 min before being added to a solution of compound 4 (2.0 g, 4.6 mmol) in *N,N*-dimethylformamide (6 mL) under argon. The reaction was stirred at room temperature for 3 hr. The solvent was removed under vacuum. Ethyl acetate (200 mL) was added to the residue and the solution was washed with 10% citric acid (100 mL x 2) followed by saturated potassium chloride (100 mL x 1). The organic layer was dried over sodium sulfate and filtered. The filtrate was concentrated in vacuo and purified by flash column chromatography, eluting with dichloromethane graded to 2.5% methanol in dichloromethane. This afforded the title compound as a foam as a mixture of two rotamers (2.75 g, 92%). δ_H (300 MHz, $CDCl_3$), (2:1 rotamers) 8.88 & 8.92 (3H, s, CONHCO), 7.96 (3H, s, NC**H**), 7.69 (6H, d, J = 7.4 Hz, Ar**H**), 7.54 (8H, m, Ar**H** & N**H**), 7.28 (12H, m, Ar**H**), 5.95 & 5.43 (3H, t, J = 7.0 Hz, N**H**Fmoc), 4.42-4.13 (15H, m, OC$\mathbf{H}_2$C**H** & C$\mathbf{H}_2$COO^{t}Bu), 4.00 & 3.89 (6H, s, NC$\mathbf{H}_2$CO), 3.52-3.28 (12H, m, NC$\mathbf{H}_2$C$\mathbf{H}_2$NHFmoc), 2.34-2.30 (6H, m, CCC$\mathbf{H}_2$), 2.10-2.18 (6H, m, C$\mathbf{H}_2$CCH), 1.90-1.87 (3H, m, CC**H**), 1.60-1.55 (12H, m, C$\mathbf{H}_2$C$\mathbf{H}_2$C$\mathbf{H}_2$CCH), 1.44 & 1.40 (27H, s, C$\mathbf{H}_3$); δ_C (75.5 MHz, $CDCl_3$): 168.7 (C), 162.8 (C), 162.1 (C), 156.7 (C), 149.9 (C), 147.2 (CH), 143.9 (C), 143.8 (C), 141.3 (C), 127.8 (CH), 127.1 (CH), 125.1 (CH), 120.0 (CH), 100.6 (C), 94.7 (C), 71.2 (C), 68.6 (C), 66.7 (CH_2), 51.1 (CH_2), 49.8 (CH_2), 48.9 (CH_2), 48.0 (CH_2), 47.3 (CH), 39.2 (CH), 28.0 (CH_3), 27.5 (CH_2), 27.4 (CH_2), 19.1 (CH_2), 17.9 (CH_2).; LRMS-ESI$^+$: m/z 653.3 $[M+H]^+$(100%).

alkyne-T-PNA monomer, 1. A solution of compound **8** (3.00 g, 4.6 mmol) in trifluoroacetic acid (9.5 mL), triisopropylsilane (0.25 mL) and water (0.25 mL) was stirred at room temperature

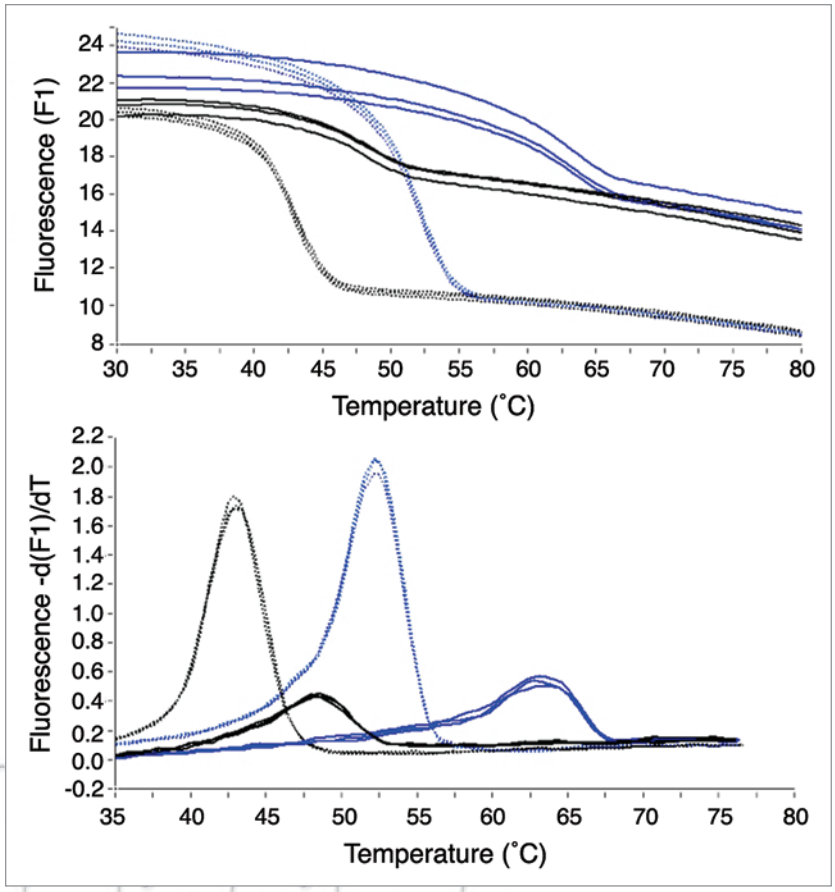


Figure 6. Comparison of melting curves and melting peaks of DNA probe (MP DNA) and PNA probe (MP3Am) with their DNA targets (PCR products of Wtt and Mtt). Top and bottom graphs present melting curves and melting peaks of MP3Am (continuous line) and MP DNA (broken line), respectively. The probes/mutant target are in blue and the probe/wild-type target are in black.

for 30 min. The mixture was then added slowly to cold diethyl ether (100 mL) and the resultant solid was filtered and dried overnight under high vacuum to give the product as an amorphous white solid as two rotamers (2.14 g, 78%). δ_H (300 MHz, DMSO-d_6), (2:1 rotamers) 12.96 (3H, br.s, COO**H**), 11.59 (3H, br.s, CONHCO), 7.88 (6H, d, *J* = 7.5 Hz, Ar**H**), 7.80 (3H, s, NC**H**), 7.73 & 7.69 (3H, br.s, CONH), 7.68 (6H, d, *J* = 7.4 Hz, Ar**H**), 7.41 (6H, dd, *J* = 7.5, 7.1 Hz, Ar**H**), 7.35 (6H, dd, *J* = 7.5 Hz, 7.1 Hz, Ar**H**), 4.71 & 4.53 (6H, s, CH_2COOH), 4.34-4.23 (9H, m, CH_2CHFmocH), 4.14 & 3.98 (6H, s, NCH_2CO), 3.41-3.11 (12H, m, NCH_2CH_2NH), 2.75 (3H, m, CC**H**), 2.34 (6H, m, CCCH_2), 2.16 (6H, br.m, CH_2CCH), 1.53 (12H, br.m, CH_2CH_2CH_2CCH); δ_C (75.5 MHz, DMSO-d_6) 170.4 (C), 166.9 (C), 162.3 (C), 156.3 (C), 150.0 (CH), 148.7 (CH), 143.9 (C), 140.7 (C), 127.6 (CH), 127.1 (CH), 125.1 (CH), 120.1 (CH), 97.9 (C), 92.8 (C), 84.2 (C), 72.7 (C), 71.4 (C), 65.5 (CH_2), 47.9 (CH_2), 47.8 (CH_2), 46.8 (CH_2), 46.7 (CH), 40.1 (CH_2), 39.0 (CH), 27.2 (CH_2), 27.1 (CH_2), 18.2 (CH_2), 17.2 (CH_2).; LRMS-ESI$^-$: *m*/*z* 595.3 $[M-H]^-$ (100). HRMS $[M+Na]^+$: *m*/*z* 619.2171; calcd. for $C_{33}H_{32}N_4NaO_7$ 619.2168.

5-[(*tert*-butoxycarbonylamino)prop-1-ynyl]uracil acetic acid, 10. To a thoroughly degassed solution of ethyl (5-iodouracil-1-yl)acetate **5** (1.99 g, 6.1 mmol) in anhydrous *N,N*-dimethylformamide (5 mL) under argon was added successively anhydrous triethylamine (7 mL), *tert*-butyl prop-2-ynylcarbamate **9** (1.66 g, 10.7 mmol), tetrakis(triphenylphosphine)palladium(0) (0.63 g, 0.5 mmol) and copper (I) iodide (0.21 g, 1.1 mmol). The mixture was left to stir at room temperature. After 2 hr the reaction was poured onto a mixture of saturated ammonium chloride (80 mL), aq. EDTA sodium salt (1.2 g in 80 mL water pH 8) and extracted with ethyl acetate (50 mL x 4). The organic fractions were combined and dried over sodium sulfate, filtered and the filtrate was concentrated in vacuo. The residue was co-evaporated with toluene (20 mL x 3), concentrated and purified by flash column chromatography eluting with 10% ethyl acetate in dichloromethane, gradually increasing to 80% ethyl acetate to give the ethyl ester of compound **10** as an amorphous off white powder (1.90 g, 90%). δ_H (300 MHz, DMSO-d_6) 11.74 (1H, s, NHCO), 8.04 (1H, s, NC**H**), 7.33 (1H, t, *J* = 5.7 Hz, NHBoc), 4.50 (2H, s, CH_2COOEt), 4.15 (2H, q, *J* = 7.2 Hz, CH_2CH$_3$), 3.92 (2H, d, *J* = 5.7 Hz, CH_2NHBoc), 1.39 (9H, s, CH_3), 1.20 (3H, t, *J* = 7.2 Hz, CH$_2$CH_3); δ_C (75.5 MHz, DMSO-d_6) 167.8 (C), 162.0 (C), 155.2 (C), 149.9 (CH), 149.1 (C), 97.4 (C), 90.3 (C), 78.2 (C), 73.4 (C), 61.3 (CH_2), 48.7 (CH_2), 30.0 (CH_2), 28.1 (CH_3), 14.0 (CH_3); LRMS-ESI$^+$: *m*/*z* 352.4 $[M+1]^+$ (100). A solution of the ethyl ester (1.93 g, 5.5 mmol) and lithium hydroxide (0.46 g, 10.9 mmol) in methanol:water (3:1, 24 mL) was stirred at room temperature for 45 min. The reaction was filtered through Dowex 50 H^+ [prewashed with 3 volumes of the following solvents: methanol, water, HCl (1 N), water, 50% pyridine in water and water] and the Dowex was washed with methanol/water (1:1 v/v, 100 mL) to fully recover the product. The eluents were combined and concentrated under high vacuum. The resultant yellow solid was washed with diethyl ether until it became white after which it was dried for 12 hr under vacuum to give compound **10** as an amorphous white solid (1.61 g, 91%). δ_H (300 MHz, DMSO-d_6) 13.23 (1H, br.s, COO**H**), 11.69 (1H, s, CONHCO), 8.03 (1H, s, NC**H**), 7.32 (1H, br.t, NHBOC), 4.42 (2H, s, CH_2COOH), 3.92 (2H, d, *J* = 4.8 Hz, CH_2NHBoc), 1.37 (9H, s, CH_3); δ_C (75.5 MHz, DMSO-d_6) 169.2 (2C), 162.1 (C), 155.2 (C), 149.9 (CH), 149.3 (C), 97.2 (C), 90.2 (C), 78.2 (C), 73.6 (C), 61.3 (CH_2), 48.7 (CH_2), 48.6 (CH_2), 30.0 (CH_2), 28.1 (CH_3); LRMS-ESI$^+$: *m*/*z* 324.5 $[M+1]^+$(100); HRMS $[M+Na]^+$: *m*/*z* 346.1013; calcd. for $C_{14}H_{17}N_3O_6Na$ 346.1010.

fully protected 5-(aminoprop-1-ynyl)uracil PNA monomer precursor, 11. A solution of the compound **10** (1.39 g, 4.3 mmol), PyBOP (2.46 g, 4.7 mmol), HOBt (0.06 g, 0.4 mmol) and *N,N*-diisopropylamine (2.2 mL, 12.9 mmol) in *N,N*-dimethylformamide (6 mL) was stirred under argon at room temperature for 5 min before being added to a solution of PNA backbone 4 (1.67 g, 3.8 mmol) in *N,N*-dimethylformamide (6 mL) under argon. The reaction was stirred at room temperature for 3 hr after which the solvent was removed under vacuum. Ethyl acetate (150 mL) was added and the resultant solution was washed with 10% citric acid (50 mL x 2) and saturated potassium chloride (50 mL x 2). The organic layer was dried over sodium sulfate, filtered and the filtrate was concentrated in vacuo before being purified by flash column chromatography using 20% ethyl acetate in dichloromethane, gradually increase polarity to 50% ethyl acetate to give compound **11** as an amorphous white powder (two rotamers). (1.711 g, 63%). δ_H (300 MHz, $CDCl_3$), (2:1 rotamers) 7.75 (9H, d, *J* = 7.4 Hz, 6ArH & 3NCH), 7.62-7.56 (8H, d, *J* = 7.4 Hz, 6ArH & 2CONHCO), 7.43-7.28 (16H, m, 12ArH, 3NHBoc & CONHCO), 6.08 & 5.61 (3H, br.t, NHFmoc), 5.01 & 4.47 (4H, br.s, CH_2COO^{t}Bu), 4.47-4.43 & 4.34 (11H, m, 3CH_2CH & 2CH_2COO^{t}Bu), 4.21 & 4.07 (6H, m, CH_2NHBoc), 4.10 & 3.95 (6H, s, CHNCH$_2$), 3.57-3.35 (12H, m, CH_2CH_2NHFmoc); δ_C (75.5 MHz, $CDCl_3$) 187.3 (C),162.1 (C), 156.7 (C), 155.4 (C), 148.6 (CH), 143.8 (C), 141.2 (C), 127.8 (CH), 127.1 (CH), 126.3 (CH), 125.2 (CH), 120.1 (CH), 99.2 (C), 90.5 (C), 83.8 (C), 82.7 (C), 80.5 (C), 73.7 (C), 66.7 (CH_2), 51.1 (CH_2), 49.8 (CH_2), 48.8 (CH_2), 48.2 (CH_2), 47.2 (CH), 39.2 (CH_2), 31.2 (CH_2), 28.4 (CH_3), 28.0 (CH_3); LRMS-ESI$^+$: *m*/*z* 702.1 $[M+H]^+$ (100).

***Fmoc*-protected 5-(aminoprop-1-ynyl)uracil PNA monomer precursor, 12.** A solution of compound **11** (1.09 g, 1.5 mmol) in trifluoroacetic acid (4.75 mL), triisopropylsilane (0.13 mL) and water (0.13 mL) was stirred at room temperature for 30 min. The resultant mixture was added slowly to cold diethyl ether (100 mL) and the solid was filtered and dried overnight under high vacuum to give the product as an amorphous white solid (two rotamers) (0.80 g, 95%). δ_H (300 MHz, $CDCl_3$), (2:1 rotamers) 6.30 (9H, d, *J* = 8 Hz, ArH & NCH), 6.16 (6H, d, *J* = 7 Hz, ArH), 5.93-5.80 (12H, m, ArH), 3.38 & 3.14 (6H, s, CH_2COOH), 2.92 & 2.77 (6H, d, *J* = 6.8 Hz, CH_2CH), 2.75 & 2.63 (3H, br.t, CH$_2$CH), 2.72 & 2.44 (6H, s, CHNCH$_2$), 2.47-2.02 (6H, m, CH_2NH$_2$), 2.03-1.83 (12H, m, CH_2CH_2NHFmoc); δ_C (75.5 MHz, $CDCl_3$) 172.3 (C),169.3 (C), 151.8 (CH), 145.3 (C), 142.6 (C), 128.8 (CH), 128.2 (CH), 126.2 (CH), 121.0 (CH), 98.2 (C), 85.7 (C), 79.6 (C), 67.9 (CH_2), 49.9 (CH_2), 49.3 (CH_2), 49.0 (CH_2), 48.5 (CH), 39.9 (CH_2), 30.8 (CH_2); LRMS-ESI$^+$:

m/z 546.1 $[M+H]^+$(95), 568.2 $[M+Na]^+$(100); HRMS $[M+Na]^+$: *m/z* 568.1802; calcd. for $C_{28}H_{27}N_5O_7Na$ 568.1803.

aminoalkyl-T-PNA monomer 2. NHS ester **13** was added to a solution of compound **12** (0.34 g, 0.6 mmol) in *N,N*-dimethylformamide (2 mL) and *N,N*-diisopropylethylamine (0.32 mL, 1.8 mmol), and the reaction mixture was stirred at room temperature for 4 hr. The solvent was concentrated in vacuo, and the product was purified by flash column chromatography using dichloromethane, gradually increase the polarity to 20% methanol in dichloromethane to give the pure compound as amorphous off white powder (rotamers) (0.29 g, 63%). δ_H (300 MHz, DMSO-d_6), (2:1 rotamers) 11.62 (3H, br.s, COO**H**), 8.28 (3H, t, *J* = 5.0 Hz, CCCH$_2$N**H**), 7.88 (6H, d, *J* = 7.4 Hz, Ar**H**), 7.85 (3H, s, CON**H**CO), 7.67 (6H, d, *J* = 7.2 Hz, Ar**H**), 7.58 (3H, br.t, N**H**Fmoc), 7.41 (6H, dd, *J* = 7.2, 7.5 Hz, Ar**H**), 7.32 (6H, dd, *J* = 7.4, 7.5 Hz, Ar**H**), 6.75 (3H, t, *J* = 6.5 Hz, N**H**Boc), 4.69 & 4.54 (6H, s, C**H**$_2$COOH), 4.32-4.20 (9H, m, C**H**$_2$C**H**), 4.03 (6H, m, CCC**H**$_2$NH), 4.86 & 3.80 (6H, s, CHNC**H**$_2$), 3.38-3.13 (9H, m, C**H**$_2$C**H**$_2$NHFmoc), 2.87 (6H, m, C**H**$_2$NHBoc), 2.79-2.73 (3H, m, CH$_2$C**H**$_2$NHFmoc), 2.06 (6H, t, *J* = 7.5 Hz, C**H**$_2$(CH$_2$)$_4$NHBoc), 1.41-1.51 (6H, m, C**H**$_2$(CH$_2$)$_3$NHBoc), 1.36 (27H, s, C**H**$_3$), 1.36-1.29 (6H, m, C**H**$_2$CH$_2$NHBoc), 1.23-1.15 (6H, m, C**H**$_2$(CH$_2$)$_2$NHBoc); δ_C (100.6 MHz, DMSO-d_6) 172.2 (C), 167.8 (C), 162.6 (C), 156.8 (C), 156.0 (C), 150.5 (C), 144.4 (C), 141.2 (C), 128.0 (CH), 127.6 (CH), 125.7 (CH), 120.5 (CH), 97.4 (C), 90.1 (C), 77.7 (C), 74.5 (C), 65.9 (CH$_2$), 49.1 (CH), 48.5 (CH$_2$), 48.2 (CH$_2$), 47.2 (CH$_2$), 38.4 (CH$_2$), 35.5 (CH$_2$), 32.6 (CH$_2$), 29.7 (CH$_2$), 28.9 (CH$_2$), 28.7 (CH$_3$), 26.5 (CH$_2$), 25.3 (CH$_2$); LRMS-ESI$^-$: *m/z* 757.2 $[M-H]^-$(100). HRMS $[M+Na]^+$: *m/z* 781.3160; calcd. for $C_{39}H_{46}N_6NaO_{10}$ 781.3168.

6-(2,2,2-trifluoroacetamido)hexyl 4-methylbenzensulfonate, 15. To a mixture of 6-amino-1-hexanol (5.00 g, 42.7 mmol) was added dimethylaminopyridine (5.20 g, 42.7 mmol) in dichloromethane (215 mL) and ethyl trifluoroacetate (10.2 mL, 85.4 mmol). The reaction mixture was stirred at room temperature and after 1 hour was concentrated under vacuum and co-evaporated with toluene (60 mL x 3) to give compound **14** which was dissolved in dichloromethane (215 mL). The mixture was cooled down to 0°C and triethylamine (11.9 mL, 85.4 mmol) and tosyl chloride (16.30 g, 85.4 mmol) were slowly added. The reaction was left to warm up to room temperature and was stirred for a further 5 hr. It was then poured onto ice, washed with water (60 mL) and sodium hydrogen carbonate (60 mL x 3), dried over sodium sulfate, concentrated and purified by flash column chromatography, eluting with toluene in hexane (0–100%) to give 6-(2,2,2-trifluoroacetamido)hexyl-4-methylbenzensulfonate **15** as an amorphous solid (11.00 g, 70%). δ_H (400 MHz, DMSO-d_6) 9.41 (1H, s, N**H**), 7.48 (2H, d, *J* = 7.5 Hz, Ar**H**), 7.12 (2H, d, *J* = 7.5 Hz, Ar**H**), 4.25 (2H, t, *J* = 6.5 Hz, C**H**$_2$OS), 3.22-3.10 (2H, m, C**H**$_2$NH), 2.29 (3H, s, C**H**$_3$), 1.69-1.59 (2H, m, C**H**$_2$CH$_2$OS), 1.54-1.16 (6H, m, C**H**$_2$C**H**$_2$C**H**$_2$CH$_2$NH); δ_C (100.6 MHz, DMSO-d_6) 155.5 (C), 145.5 (C), 137.7 (C), 128.0 (2CH), 125.4 (2CH), 76.0 (CH$_2$), 38.9 (CH$_2$), 28.9 (CH$_2$), 28.0 (CH$_2$), 25.5 (CH$_2$), 24.2 (CH$_2$), 20.7 (CH$_3$); ^{19}F NMR (DMSO-d_6): *d* ppm -74.35 (CF$_3$CO-); MS-ESI$^+$: *m/z* 390.2 $[M-H]^-$.

***N*-(6-azidohexyl)-2,2,2-trifluoroacetamide, 16.** To a mixture of 6-(2,2,2-trifluoroacetamido)hexyl-4-methylbenzensulfonate **15** (5.60 g, 15.2 mmol) in anhydrous *N,N*-dimethylformamide (40 mL) was added potassium carbonate (3.20 g, 22.8 mmol) and sodium azide (2.0 g, 30.4 mmol). The mixture was stirred at 50°C for 5 hr, filtered through a pad of celite and the solvent was removed in vacuo. The residue was co-evaporated with toluene (20 mL x 2), concentrated and purified by flash column chromatography, eluting with toluene in hexane (0–100%) to give *N*-(6-azidohexyl)-2,2,2-trifluoroacetamide **16** as a yellow oil (3.40 g, 94%). δ_H (400 MHz, DMSO-d_6) 9.36 (1H, s, N**H**), 3.31 (2H, t, *J* = 6.8 Hz, C**H**$_2$N$_3$), 3.21-3.13 (2H, m, C**H**$_2$NH), 1.57-1.44 (4H, m, C**H**$_2$CH$_2$NH and C**H**$_2$CH$_2$N$_3$), 1.36-1.22 (4H, m, C**H**$_2$C**H**$_2$(CH$_2$)$_2$N$_3$); δ_C (400 MHz, DMSO-d_6) 155.0 (C),114.8 (C), 50.4 (CH$_2$), 39.0 (CH$_2$), 28.0 (CH$_2$), 27.9 (CH$_2$), 25.57 (CH$_2$), 25.52 (CH$_2$); δ_F (DMSO-d_6) -74.35 (CF$_3$CO-); MS-ESI$^+$: *m/z* 237.2 $[M+H]^+$; HR-ES $[M+Na]^+$: *m/z* 261.0937; calcd. for $C_8H_{13}F_3N_4NaO$ 261.09392.

6-azido-1-aminohexane, 17. *N*-(6-azidohexyl)-2,2,2-trifluoroacetamide **16** (3.30 g, 14.0 mmol) was dissolved in aqueous ammonia solution (35%, 500 mL) and the reaction mixture was stirred for 12 hr at room temperature. It was then concentrated to dryness, co-evaporated with methanol (30 mL x 2), redissolved in aqueous methanol (50:50) and passed through a column of Dowex H$^+$ form 50W (500 mL) eluting with 3% aqueous ammonia in methanol (1:1). The eluent was collected, the solvent was evaporated and the product was dried under vaccuum overnight to give 6-azido-1-aminohexane **17** as a yellow oil (1.50 g, 76%). δ_H (400 MHz, DMSO-d_6) 7.77 (s, 2H, N**H**$_2$), 3.37-3.30 (2H, obsc by residue peak of DMSO, CH$_2$C**H**$_2$N$_3$), 2.81-2.71 (2H, t, *J* = 7.5 Hz, C**H**$_2$NH$_2$), 1.59-1.46 (4H, m, C**H**$_2$(CH$_2$)$_2$CH$_2$C**H**$_2$N$_3$), 1.35-1.28 (4H, m, C**H**$_2$C**H**$_2$(CH$_2$)$_2$N$_3$); δ_C (400 MHz, DMSO-d_6) 50.4 (CH$_2$), 38.8 (CH$_2$), 27.9 (CH$_2$), 26.7 (CH$_2$), 25.5 (CH$_2$), 25.2 (CH$_2$); MS-ESI$^+$: *m/z* 143.2 $[M+H]^+$; HR-ES $[M+H]^+$: *m/z* 143.1293; calcd. for $C_6H_{15}N_4$ 143.12967.

di-*O*-pivaloyl-6-[fluorescein-5-carboxamido]azidohexane, 19. To a solution of di-*O*-pivaloyl-5(6)-carboxyfluorescein **18** (0.66 g, 1.2 mmol) in anhydrous dichloromethane (12 mL) was added HOBt (0.32 g, 2.4 mmol) and EDC (0.46 g, 2.4 mmol). The reaction mixture was stirred under argon for 5 min, then 6-azido-1-aminohexane **17** (0.17 g, 1.2 mmol) was added. After 1 hour the reaction mixture was concentrated and purified by flash column chromatography, eluting with ethyl acetate in hexane (0–40%) to give di-*O*-pivaloyl-6-[fluorescein-5-carboxamido] azidohexane **19** as a white foam (190 mg, 24%). A small quantity of the impure 6-isomer was also isolated. δ_H (400 MHz, $CDCl_3$) 8.46 (1H, br. s, Ar**H**), 8.23 (1H, d, *J* = 8.0 Hz, Ar**H**), 7.26 (1H, d, *J* = 8.0 Hz, Ar**H**), 7.07 (2H, br. s, Ar**H**), 6.78 (5H, br. s, Ar**H** & N**H**), 3.48 (2H, t, *J* = 6.7 Hz, C**H**$_2$NH), 3.23 (2H, m, C**H**$_2$N$_3$), 1.70-1.53 (4H, m, 4H, C**H**$_2$(CH$_2$)$_2$C**H**$_2$ CH$_2$NH), 1.45-1.37 (4H, m, C**H**$_2$CH$_2$(CH$_2$)$_2$NH), 1.35 (18H, s, C**H**$_3$); δ_C (100.6 MHz, $CDCl_3$) 176.4 (2C), 168.6 (C), 165.4 (C), 155.1 (C), 152.8 (C), 151.6 (C), 137.1 (C), 135.1 (CH), 128.7 (2CH), 126.4 (C), 124.5 (CH), 123.1 (CH), 117.8 (2CH), 115.5 (2C), 110.4 (2CH), 82.3 (C), 51.3 (CH$_2$), 40.2 (CH$_2$), 39.2 (2C), 29.3 (CH$_2$), 28.7 (CH$_2$), 27.0 (CH$_3$), 26.4 (CH$_2$), 26.3 (CH$_2$);

MS-ESI$^+$: *m/z* 691.8 [M+Na]$^+$; HR-ES [M+Na]$^+$: *m/z* 691.2753; calcd. for $C_{37}H_{40}N_4NaO_8$ 691.27438.

5-Azidohexylfluorescein 3. di-*O*-pivaloyl-6-[fluorescein-5-carboxamido]azidohexane **19** (0.30 g, 0.4 mmol) was dissolved in 1,4-dioxane (30 mL) and concentrated aqueous ammonia (35%, 60 mL) was added. After 3.5 hr the reaction mixture was concentrated, co-evaporated with methanol (30 mL x 3) and purified by flash column chromatography, eluting with methanol in dichloromethane (0–30%) with 0.5% trifluoroacetic acid. The product was dissolved in methanol and precipitated by addition of water (15 mL). Drying overnight under vacuum gave compound **3** as an amorphous yellow powder (0.19 g, 93%). δ_H (400 MHz, DMSO-d_6) 10.14 (1H, s, COOH), 8.79 (1H, t, *J* = 5.4 Hz, N**H**), 8.47 (1H,br. s, Ar**H**), 8.25 (1H, d, *J* = 8.1 Hz, Ar**H**), 7.37 (1H, d, *J* = 8.1 Hz, Ar**H**), 7.00 (1H, br. s, O**H**), 6.70 (1H, s, Ar**H**), 6.69 (1H, s, Ar**H**), 6.61-6.52 (4H, m, Ar**H**), 3.39-3.27 (4H, m, C**H**$_2$NH & C**H**$_2$N$_3$), 1.62-1.49 (4H, m, C**H**$_2$CH$_2$NH & C**H**$_2$CH$_2$N$_3$), 1.40-1.31 (4H, m, C**H**$_2$C**H**$_2$(CH$_2$)$_2$NH); δ_C (100.6 MHz, DMSO-d_6) 168.1 (C), 164.4 (C), 159.5 (2C), 154.5 (C), 151.8 (2C), 136.3 (CH), 134.6 (CH), 129.0 (2CH), 126.4 (C), 124.1 (C), 123.1 (CH), 112.6 (2CH), 109.0 (2C), 102.2 (2CH), 83.2 (C), 50.5 (CH$_2$), 37.8 (CH$_2$), 28.7 (CH$_2$), 28.1 (CH$_2$), 25.9 (CH$_2$), 25.8 (CH$_2$); MS-ESI$^-$: *m/z* 499.3 [M-H]$^-$; HR-ES [M+H]$^+$: *m/z* 501.1762; calcd. for $C_{27}H_{25}N_4O_6$ 501.17741.

Synthesis, purification and analysis of PNA probes. Standard Fmoc PNA monomers and Fmoc Xal resin were purchased from Link Technologies Ltd.; piperidine and DMF (peptide synthesis grade) were purchased from Rathburn; HATU and amino acids were bought from Novabiochem and CEM Ltd.; DIPEA, 2,6-lutidine, acetic anhydride, trifluoroacetic acid (TFA) and triisopropylsilane (TIS) were bought from Sigma Aldrich. All the PNA oligomers were synthesized on an Expedite Nucleic Acid/PNA synthesiser on the 2 μmole scale. Cleavage and deprotection of the PNA was carried out using TFA:water:TIS in a ratio of 95:2.5:2.5 (400 μL/2 μmole) for 20 min. Purification of PNA was carried out by reversed-phase HPLC on a Gilson system using an Ascentis C18 (Supelco) column, 10 mm x 250 mm, pore size 10 micron. The following protocols were used: Run time 35 min, flow rate 3 mL per min, binary system, gradient: Time in min (% buffer B); 0 (5); 5 (5); 24 (30); 26 (95); 29 (95); 30 (5); 35 (5). Elution buffer A: 0.1% TFA in water and buffer B: 0.08%TFA in acetonitrile. Elution of PNAs was monitored by ultraviolet absorption at 295 nm. PNAs were then freeze dried and re-dissolved in water at working concentration. The molecular mass of the PNAs was verified by a Low Resolution MALDI-TOF MS on a Waters (micromass) TofSpec2E 337 nm laser operating in linear mode with external calibration of ±0.1%.

General procedure for solution phase click chemistry. A solution of $CuSO_4$ in water (100 nmol/μL) was degassed by purging with argon gas and added to a degassed solution of sodium ascorbate (500 nmol/μL) in water. The mixture was degassed one more time before being added to a degassed solution of PNA in water. The 5-azidohexylfluorescein **3** in THF was added to the reaction mixture and the resultant mixture was degassed and left in the dark at room temperature for 2 hr. THF 1 mL was added and the solution was centrifuged at 5,000 rpm for 2 min. The supernatant was removed and the pellet was re-dissolved in 0.1% TFA in water. The product was purified by reverse phase HPLC. Note: The final concentration of PNA in the reaction was less than 0.2 mM, the ratio of water to THF was ~1:1). The molar ratio of PNA:$CuSO_4$:sodium ascorbate:dye was 1:10:100:50.

General procedure for solid phase click chemistry. Use similar condition as for solution phase labelling, a solution of $CuSO_4$ was added to a degassed solution of sodium ascorbate. The reaction was degassed one more time before being added to the PNA resin in DMF under argon. Then a solution of degassed dye in DMF was added to the reaction mixture under argon and the mixture was left at room temperature for 2 hr. The resin was transfer to an ALL-FIT column (Link Technologies Ltd.,) and washed with DMF (4 x 1 mL), DCM (3 x 1 mL) and diethyl ether (2 x 1 mL). The resin was dried under a stream of argon gas for 10 min before cleavage using the same conditions as for the PNA probe synthesis.

PCR amplification and fluorescence melting analysis. Target amplification and melting curve analysis was performed using a Roche LightCycler® 1.5 instrument in single experiment. Reactions were performed in 10 μL reaction volumes, comprising 0.5 μM of reverse primer (RP), 0.05 μM of forward primer (FP), 0.5 mM dNTPs, 0.1 μM of the probe, 3.0 mM of $MgCl_2$, 10 ng/μL BSA (Roche Diagnostics, Lewes, UK), 0.5 Units HotStarTaq (QIAGEN, Crawley, UK) and 4 ng of the DNA targets. The PCR thermal cycling conditions were 95°C for 15 min to activate the enzyme followed by 35 cycles of 94°C/10 sec, 50°C/15 sec and 72°C/15 sec. Following amplification, reactions were heated to 95°C for 30 sec and cooled at 30°C for 1 min. Melting curve analysis was then performed by heating reactions from 30°C to 95°C at 0.1°C/sec. The fluorescence melting curve data were converted to the first derivatives giving the melting peaks (-d(F)/dT where F is fluorescence and T is temperature in °C). The total time of the amplification and melting curve analysis was on average 60 min and depended on the ambient temperature, i.e., ability of LightCycler® to cool to 30°C.

Oligonucleotide synthesis and purification. Oligonucleotides, including HyBeacons, were synthesized by standard methods.[20]

Conclusions

Fluorescein-labelled PNA HyBeacons have been synthesised by two methods; by click chemistry to create a triazole linkage, and by NHS ester-amine coupling for amide bond formation. In principle these approaches could be combined to offer orthogonality for labelling PNA with different dyes or other reporter groups at specific loci in the sequence. PNA HyBeacons give superior mismatch discrimination to DNA HyBeacons but much smaller fluorescence change on melting. The origin of the weaker fluorecence change is under investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors wish to thank Julie M. Herniman for helping with MS analyses.

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